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Efficiency of Neutralizing Antibodies Targeting the CD4-Binding Site: Influence of Conformational Masking by the V2 Loop in R5-Tropic Clade C Simian-Human Immunodeficiency Virus[▽]

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In R5-tropic clade C simian-human immunodeficiency viruses (SHIV-Cs), we identified a 3-asparagine (3N) deletion mutation in the V2 loop stem of gp120 as the major determinant of neutralization escape of the anti-CD4-binding site (anti-CD4-bs) neutralizing monoclonal antibody (nMAb) b12. However, the more potent anti-CD4-bs nMAbs VRC01 and VRC03 were not sensitive to this mutation. Using isogenic tier 1 or tier 2 proviruses differing only in the 3N mutation, we showed that this mutation might result in selective conformational b12 epitope masking. Therefore, human immunodeficiency virus (HIV) Env immunogens targeting the CD4-bs and designed to neutralize tier 2 viruses should take conformational masking by the V2 loop into account.

Passive immunization studies with neutralizing monoclonal antibodies (nMAbs) gave proof of concept that nMAbs targeting epitopes on the Env protein can prevent simian-human immunodeficiency virus (SHIV) acquisition (1, 6, 8, 14, 15, 17, 23). However, designing immunogens capable of inducing potent humoral responses that neutralize the majority of human immunodeficiency virus (HIV) strains remains a challenge.

The CD4-binding site (CD4-bs) represents an attractive target since gp120 binds to host cells via the CD4 receptor to promote viral entry (5). Several anti-CD4-bs nMAbs have been isolated: the IgG1 b12, HJ16, VRC01, and VRC03 (2, 3, 24). All of these nMAbs recognize different epitopes that overlap with the CD4-bs, resulting in different neutralization potencies. The recently isolated nMAb VRC01 was able to neutralize 90% of the viruses tested, resulting in a neutralization breadth exceeding that of b12. Therefore, it is important to understand the differences in neutralization mechanisms between VRC01 and b12. Among gp120 features that could help the virus evade humoral immune responses, the V2 loop has been shown to be involved in the conformational masking of epitopes (11, 13, 18, 25).

Two R5-tropic clade C SHIVs (SHIV-Cs) that carry *env* related to a pediatric HIV clade C (HIV-C) isolate, HIV1157i, have been developed by our laboratory and used in challenge studies (9, 10, 23). SHIV-1157ipEL-p carries the recently transmitted *env* and has a tier 1 neutralization profile (20). SHIV-1157ipd3N4, the late form (21), was reisolated when a rhesus monkey (RM), chronically infected with the parental SHIV-1157i, had progressed to AIDS; SHIV-1157ipd3N4 is more neutralization resistant, with a tier 2 neutralization profile. A longer V1V2 loop and/or an increased number of potential N-glycosylation (PNG) sites have been linked to neutralization escape (22). Interestingly, the late SHIV-1157ipd3N4 has a shorter V2 loop, due to a deletion of 3 asparagines (3N) in the V2 stem, and one PNG site less than the early SHIV-1157ipEL-p (Fig. 1). Consequently, neutralization escape could not be due to a longer and/or more glycosylated V2 loop in our SHIV-Cs but is more likely due to a different position of the V2 loop. We hypothesized that the 3N deletion in the V2 stem was modifying the position of the V2 loop, resulting in conformational masking of CD4-bs epitopes. Using molecular modeling in combination with site-directed mutagenesis, we found that the different position of the V2 loop impaired the neutralization by b12 but not by VRC01. We conclude that the neutralization potency of VRC01 is due to its ability to avoid conformational masking or steric hindrance of its epitope by the V2 loop in our SHIV-C model.

Two SHIV-C mutants were designed: a mutant of the early SHIV-1157ipEL-p, termed SHIV-1157ipEL-pΔ3N, which lacked the 3N residues in the V2 stem, and a mutant of the late SHIV-1157ipd3N4, termed SHIV-1157ipd3N4+3N, where we added 3N residues in the V2 stem (Fig. 1). The infectious molecular clones of SHIV-1157ipd3N4+3N and SHIV-1157ipEL-pΔ3N were constructed by overlapping PCR, and virus stocks were generated in RM peripheral blood mononuclear cells. These four SHIV-Cs were isogenic, as they were cloned in the same engineered backbone (21) and differed only by the specific mutation in the V2 stem.

Next, we compared the sensitivities of the early/late SHIV-Cs and their mutants to the anti-CD4-bs nMAbs b12, VRC01, VRC03, and HJ16 and to soluble CD4 (sCD4) by TZM-bl assay (16). sCD4 neutralized the four SHIV-Cs with no significant differences and 50% inhibitory concentrations (IC_{50}s) ranging from 1.51 to 5.48 μg/ml ($P = 0.207$) (Fig. 2A and B). While the early SHIV-1157ipEL-p was neutralized by b12 (IC_{50} of 1.59 μg/ml), its mutant SHIV-1157ipEL-pΔ3N was not, even at a high concentration (40 μg/ml) ($P < 0.0001$).

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SHIV-1157ipEL-p (early)     CSNFAREGNVTYKEEMDKVKNCSFNVTTGIRDKKQKVNALFYRLDITPLDENNNNSSEYKLINC
SHIV-1157ipd3N4 (late)      ....TGKS.....GD.E-..........E........Y...........D---.....I....
SHIV-1157ipEL-pΔ3N          ..........................................................---.........
SHIV-1157ipd3N4+3N          ....TGKS.....GD.E-..........E........Y...........DNNN.....I....
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FIG. 1. Sequence alignment of the V1V2 loop of SHIV-1157ipEL-p (early stage), carrying the recently transmitted *env* of the Zambian clade C isolate 1157i, and its mutant SHIV-1157ipEL-pΔ3N, as well as SHIV-1157ipd3N4 (late stage) and its mutant SHIV-1157ipd3N4+3N. The black box highlights the insertion/deletion of 3 asparagines in the V2 stem.

Moreover, b12 did not neutralize the late SHIV-1157ipd3N4 but neutralized the mutant SHIV-1157ipd3N4+3N (IC_{50} of 0.93 μg/ml) ($P < 0.0001$). However, VRC01 neutralized all four viruses, with IC_{50}s ranging from 0.74 to 3.17 μg/ml and no significant differences ($P = 0.095$) (Fig. 2C and D). VRC03 also neutralized the four SHIV-Cs (IC_{50}s ranging from 0.282 to 6.68 μg/ml) ($P = 0.261$) (Fig. 2C and D). Therefore, both VRC01 and VRC03 avoid the conformational masking by the V2 loop in SHIV-Cs. Furthermore, nMAb HJ16 neutralized neither early nor late SHIV-Cs (data not shown), indicating that the HJ16 epitope may not be present on the SHIV-C envelopes.

To confirm these results, we performed a virion capture assay (Fig. 3). Viruses (p27, 400 ng/ml) were added to nMAbs and incubated for 20 h at 37°C. After being washed, the plates were incubated with 0.5% Triton X-100 for 1 h, followed by simian immunodeficiency virus (SIV) p27 capture assay (ABL, Inc.). The human IgG1 MAb Herceptin was used as a negative isotype control. Indeed, b12 was able to bind to the early SHIV-1157ipEL-p but not to its mutant SHIV-1157ipEL-pΔ3N ($P = 0.001$). Furthermore, b12 did not bind to the late SHIV-1157ipd3N4 but bound to its mutant SHIV-1157ipd3N4+3N ($P = 0.002$). The results of the virion capture assay agree with those of the neutralization experiments, indicating that the 3N deletion in the V2 stem of the late SHIV-1157ipd3N4 impaired binding as well as neutralization by b12. In contrast, VRC01 was able to bind to all four SHIV-Cs, confirming that VRC nMAbs, like sCD4, were not sensitive to the conformational masking by the V2 loop. We conclude that V2 loop orientation, and mostly the insertion/deletion of 3N residues, is sufficient to induce b12 neutralization escape in our SHIV-Cs and that conformational masking by the V2 loop regulates access to the b12 epitope but not to VRC epitopes.

Then, we sought to compare b12 and VRC01 epitopes on SHIV-C gp120. We used published gp120 structures to model SHIV-1157ipEL-p gp120 in complex to b12 (Fig. 4A) and VRC01 (Fig. 4B). The V2 loop was not modeled due to the lack of structural data. Protein modeling and energy calculations were performed using Discovery Studio (Accelrys Software, Inc.) based on a sequence alignment with the core struc-

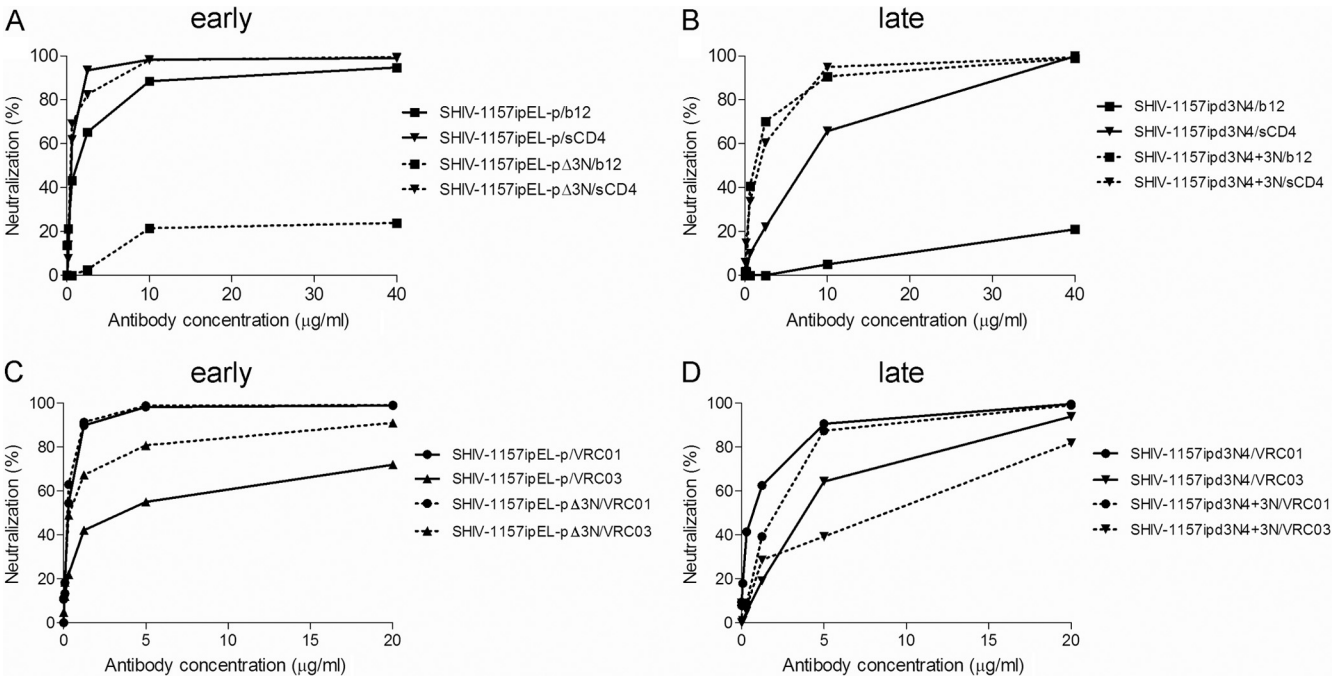

FIG. 2. Neutralization sensitivities of SHIV-1157ipEL-p (early stage) and its mutant SHIV-1157ipEL-pΔ3N to b12 and sCD4 (A) and SHIV-1157ipd3N4 (late stage) and its mutant SHIV-1157ipd3N4+3N to b12 and sCD4 (B) and of SHIV-1157ipEL-p (early stage) and its mutant SHIV-1157ipEL-pΔ3N to VRC01 and VRC03 (C) and SHIV-1157ipd3N4 (late stage) and its mutant SHIV-1157ipd3N4+3N to VRC01 and VRC03 (D). The data shown are representative results obtained from three independent experiments.

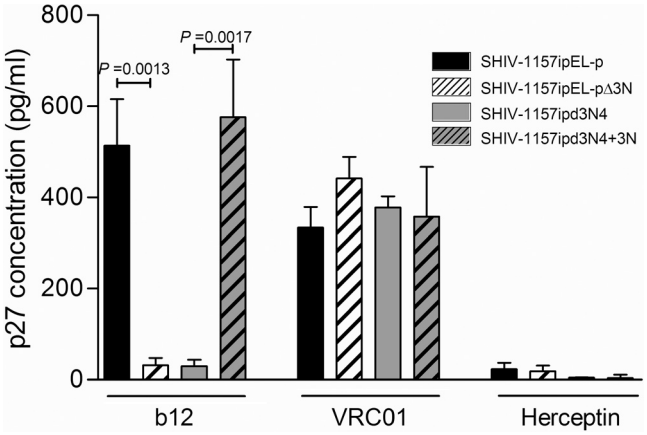

FIG. 3. Virion capture assay of SHIV-1157ipEL-p (early stage) and its mutant SHIV-1157ipEL-pΔ3N and SHIV-1157ipd3N4 (late stage) and its mutant SHIV-1157ipd3N4+3N, using nMAbs b12 and VRC01. Herceptin is used as a negative control. The error bars represent the standard deviations measured in one experiment carried out in triplicate. The data shown are representative results obtained from three independent experiments.

ture of gp120 in b12-bound conformation (Protein Data Bank [PDB] code 2NY7) (26) and VRC01-bound conformation (PDB code 3NGB) (27). Energies were calculated using CH ARMM (Chemistry at Harvard Macromolecular Mechanics). We introduced solvent factors using the implicit of distance dependent dielectrics model and performed energy minimization (steepest descent following by conjugate gradient). nMAb b12 interacts mainly with the outer domain of gp120, but the position of the V2 loop may mask the b12 cognate epitope, due to steric hindrance. The 3N deletion in the V2 stem influences the position of the V2 loop (Fig. 4A and B) and generates a steric hindrance. For VRC01, however, the movement of the V2 loop resulting from the deletion in the V2 stem does not mask the cognate epitope, implying that VRC01 is less sensitive than b12 to conformational masking by the V2 loop.

It has been shown that the length of the V2 loop could change over the course of infection (4, 22). Indeed, a longer and/or more glycosylated V2 loop has been linked to an increase in neutralization resistance (22). However, our SHIV-Cs differ because the late SHIV-1157ipd3N4, although more neutralization resistant than the early SHIV-1157ipEL-p, has a shorter V2 loop. We believe that the virus adapts its V2 loop's length, either shorter or longer, to allow different positioning of the V2 loop, thus creating steric hindrance of neutralizing epitopes. Thus, the epitope is no longer accessible on the viral spike due to conformational masking. The b12 epitope is present in our early and late SHIV-Cs, but the position of the V2 loop masks the epitope in the late SHIV-1157ipd3N4. This is in agreement with a recent study that identified a b12 amino acid residue signature in the V2 stem (7). However, VRC01 avoids the conformational masking by the V2 loop, as postulated previously (27). Recently isolated anti-CD4-bs nMAbs have been shown to share common features with VRC01 that could explain their impressive potency (19). We postulate that these nMAbs may also avoid the conformational masking by the V2 loop. However, VRC01 neutralization is dependent on mutations in the V5 loop (12, 27).

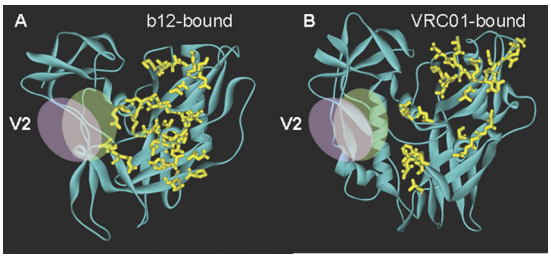

FIG. 4. Molecular modeling of SHIV-1157ipEL-p in b12-bound conformation, with contact residues between b12 and gp120 shown in yellow (A), and of SHIV-1157ipEL-p in VRC01-bound conformation, with contact residues between VRC01 and gp120 shown in yellow (B). In both conformations, the potential location of the V2 loop is indicated in pink for SHIV-1157ipEL-p (early) and in green for SHIV-1157ipd3N4 (late).

To generate broadly protective AIDS vaccine candidates, CD4-bs epitopes represent an attractive immunogen. However, given that the V2 loop is one of the most mutated Env regions, the conformational masking by the V2 loop should be avoided to retain optimal exposure of the conserved CD4-bs domain, making the VRC01 epitope appealing for immunogen development.

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